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Haptotactic Migration of Pancreatic Cancer Cells Induced by Bioactive Components in Bovine Liver Extract

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Background and Objectives: Migration into subendothelial tissue in remote organ by cancer cells are crucial events for organ-specific metastasis, including liver metastasis. This study aims to investigate the chemoattractive ingredients in liver extract, which induce migration of liver metastatic cancer cells.

Methods: Cell migrations of SU.86.86 cells, human pancreatic cancer cells raised from liver metastasis, toward the bovine liver extract were studied by chemotaxis or haptotaxis assay. Bovine liver extract was partially purified by chromatographic or gel elutriation method.

Results: The soluble fraction of the liver extract did not induce chemotactic migration of SU.86.86 cells. However, the insoluble fraction induced a remarkable haptotactic migratory response. C_{18} column unbound insoluble fractions eluted from SDS-PAGE induced chemotactic migration of SU.86.86 cells.

Conclusion: Such bioactive components in liver extract may play an important role in the development of liver metastasis.

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KEY WORDS: organ-specific metastasis; liver metastasis; chemotaxis; haptotaxis

INTRODUCTION

The liver is a favorite target site for the metastatic spread of many cancers, particularly those of the gastrointestinal tract, including pancreas cancer. It is obvious that the presence of liver metastasis carries a very poor prognosis. The selective colonization of certain organs by metastatic cells was first described in 1889 by Paget [1], who theorized that metastasis formation was a consequence of specialized tumor cells (''seed'') finding the correct environment (''soil'') for arrest, invasion, and growth. As the factor of ''soil,'' the organ site for metastatic colonization possesses the proper type of endothelium, organ matrix, and paracrine growth factors.

Incidentally, during the process of cancer invasion a number of tissue barriers, such as basement membrane and surrounding connective tissue, have to be degraded [2–4]. Invading tumors degrade these natural barriers by releasing proteases. The degraded extracellular matrix releases the soluble chemoattractant for cancer cell migration (chemotaxis), or work themselves as the chemoattractant for promoting tumor invasion and growth (chemotaxis or haptotaxis) [5]. Chemoattractants include various growth factors, the extracellular matrix components, and some proteases present in the tissue [6].

This study shows that chemoattractants for human metastatic cells are present in bovine liver extracts. Those chemoattractive ingredients exist in the crude insoluble fraction of the liver extracellular matrix extracted with 4 M guanidine. The liver-metastatic pancreatic can-

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cer cells did not show any chemotactic response to soluble liver ingredients, but they demonstrated remarkable haptotactic response to insoluble liver ingredients. The insoluble haptotactic ingredients may not act as chemoattractant for cancer cells until the cells invade the extracellular matrix. They may only behave as attractants when cancer cells invade the liver stroma, breaking through the endothelium of vessels. The cancer cells that have left the original organ reach and attach to the endothelium of a remote organ. It is speculated that these insoluble bioactive components play an important role in the final step of cancer metastasis by inducing haptotaxis of cancer cells, after the attachment of the endothelium cells of a remote organ.

MATERIALS AND METHODS

Cells

A human metastatic pancreatic carcinoma cell line, SU.86.86 (American Type Culture Collection [ATCC] (Rockville, MD) CRL1837), was raised from metastatic lesion of liver. Cells were maintained in RPMI-1640 supplemented with 10% fetal bovine serum (FBS), 100 μg/ml gentamicin (RPMI-FBS medium).

Preparation of Tissue Extracts

Bovine liver was homogenized with Ultra-Turrax in 0.5 M NaCl, 50 mM Tris HCl, pH 7.5, containing 5 mM ethylenediaminetetraacetic acid (EDTA), 5 mM phenylmethane sulfonyl fluoride (PMSF), and 5 mM N-ethylmaleimide (NEM), and extracted for 24 h at 4°C in the same buffer. Extracts were centrifuged at 12,000*g* for 30 min, and the supernatant fraction was dialyzed against water and lyophilized. The lyophilized soluble fraction of the crude liver extract was stocked as soluble liver extract. The pellet was re-extracted for 48 hr at 4°C in 4 M guanidine–HCl, 0.5 M NaCl, 50 mM Tris–HCl, pH 7.5 containing 5 mM EDTA, 5 mM PMSF, and 5 mM NEM. Extracts were centrifuged at 45,000*g* for 2 hr, and the supernatant fraction was dialyzed against water and lyophilized. Lyophilized crude liver extract was stocked as insoluble liver extract; this fraction was not completely dissolved in ordinary buffer or culture media such as phosphate-buffered saline (PBS) or RPMI-1640.

Chemotaxis Assay

Chemotaxis was assayed using a 48-well chemotaxis chamber (Neuro Probe, Cabin John, MA) and 8-mm Nucleopore filters (Pleasanton, CA) coated with 1.0 mg/filter type IV collagen. Tumor cells (2×10^5) in serum-free RPMI-1640 were added to the upper compartment of the chamber; the lower wells contained serum-free RPMI-1640 as a control and liver extract dissolved in serum free RPMI-1640. The chamber was incubated for 4 hr in a humidified 5% CO_2 incubator at 37°C. After 4 hr of incubation, the filter was removed, fixed in 100% methanol for 10 min, and stained with hematoxylin and eosin (H&E). The filter was washed in distilled water and mounted on glass slides. The surface of the upper well side was wiped clean with a cotton swab to remove the cell pellets that did not penetrate the filter. Cells that had migrated to the lower side of the filter were counted by light microscopy under high-power fields (×200). Results were expressed as the number of migrated cells and represented as the mean ± standard deviation (±SD) of five experiments, each assayed in quadruplicate. The amount of protein was determined by the Bradford [7] assay, using Bio-Rad Protein Assay reagent (Bio-Rad, Hercules, CA).

Haptotaxis Assay

Haptotaxis was assayed using the same 48-well chemotaxis chamber as in the chemotaxis assay and 8-mm Nucleopore filters coated with the insoluble liver fraction prepared by means of the following procedures. Lyophilized crude insoluble liver fraction was dissolved in 0.5 M acetic acid. Filter coating for haptotaxis was carried out according to Klominek et al. [5] with some modifications. The uncoated filter was placed in the chamber, the dull side of the filter facing the upper wells. The upper wells were filled with different concentrations of liver extracts in 0.5 M acetic acid, and the lower wells remained empty. The chamber was left overnight at 4°C; the filter was then removed and washed on both sides, first in PBS, then in distilled water, and air dried. The dried filter was placed in the 48-well chemotaxis chamber with the coated spots of the filter facing exactly toward the lower wells, which were filled with serum-free RPMI medium. The cell motility assay was performed according to the same procedures as the chemotaxis assay.

Separation of Insoluble Fraction by C_{18} Column

The lyophilized insoluble fraction of crude liver extract was dissolved in 0.1% trifluoroacetic acid (1 mg/ml) and separated with a C_{18} Sep-Pak cartridge (Millipore, Marlborough, MA) by the method of reverse phase chromatography. The C_{18} bound fraction was eluted with 60% and 100% acetonitrile. Elutriated C_{18} bound and unbound fractions were desiccated by Speed Vac (Savant, NY).

SDS-PAGE and Gel Elutriation

Crude insoluble fractions of liver extract dissolved in sample loading buffer were separated on an 8% polyacrylamide gel under reduced conditions by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After electrophoresis of the C_{18} unbound fraction, the gel was divided into six oblong slices according to molecular weight: <40 kD, 40–60 kD, 60–80 kD, 80–120 kD, 120–210 kD, and >210 kD, respectively. Protein

elutriation from the gel slice was performed for 120 min at 50 V using electrolytic gel eluter GE200 (Pharmacia Biotech, Uppsala, Sweden). After the elution, each sample was dialyzed against water and desiccated by Speed Vac. Six protein fractions (Fr. 1–Fr. 6) were obtained from the six gel slices, and the molecular weight range of each fraction was <40 kD, 40–60 kD, 60–80 kD, 80–120 kD, 120–210 kD, and >210 kD, respectively. Each fraction was dissolved in 100 μl of serum-free RPMI-1640 medium and serially diluted with same medium. The amount of protein was determined by the Bradford assay [7].

RESULTS

Chemotaxis and Haptotaxis Assay for Crude Liver Extract

Lyophilized soluble liver extract was added to the lower compartment of the chemotaxis chamber with concentration from 0.01 μg/ml to ⩽1,000 μg/ml. SU.86.86 cells showed no chemotactic response to the soluble liver extract (Fig. 1A). On the other hand, crude insoluble liver extract coated on the lower side of filters (0.01–10,000 μg/ml) induced haptotactic migratory response for SU.86.86 cells (Fig. 1B). The graph chart of haptotaxis was not linear and appeared to be composed of several parts. Even in a very low level of insoluble liver extract (0.01 μg/ml), SU.86.86 cells significantly migrated toward the other side of the filter, compared with the control.

Haptotaxis for C_{18} Column Separated Insoluble Liver Extract

In order to investigate the chemoattractants in insoluble liver extract, which was only soluble in acidic solution such as acetic acid, the extract was dissolved in 0.1% trifluoroacetic acid and separated with a C_{18} column. The C_{18} bound and unbound fraction of insoluble liver extract induced haptotactic migratory response of the SU.86.86 cells (Fig. 2A,B) SU.86.86 substantially migrated toward the C_{18} bound fraction biphasically (Fig. 2A) and toward the C_{18} unbound fraction parabolically in a concentration-dependent manner (Fig. 2B).

SDS-PAGE

The sample buffer for SDS-PAGE was able to dissolve the insoluble liver extract. The C_{18} unbound fraction of insoluble liver extract separated on the 8% SDS-PAGE gel; however, it contained several dark bands stained with Coomassie blue dye in reduced condition, composed mainly of low-molecular-weight components (Fig. 3A).

Chemotaxis for C_{18} Unbound Fraction Eluted From Gel

Protein fractions eluted from the SDS-PAGE of the C_{18} unbound fraction were soluble in RPMI-1640 medium. The elutriated protein fractions of Fr. 1, Fr. 3, Fr. 4, and Fr. 5 induced chemotactic migratory response of SU.86.86 cells, while Fr. 2 and Fr. 6 induced no migratory response (Fig. 3B). Each eluted fraction (except Fr. 2) contained an almost equal amount of protein; protein concentrations of Fr. 1–Fr. 6 were 20.54, 1.33, 19.41, 16.18, 19.76, and 21.42 μg/ml, respectively.

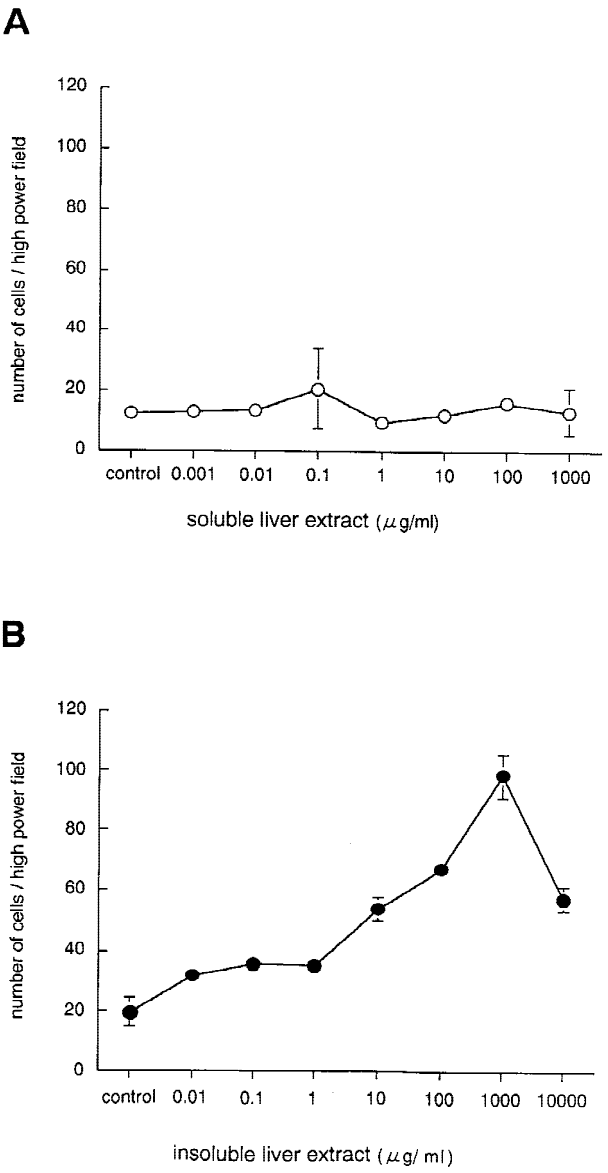

Fig. 1. **(A)** Chemotactic response of SU.86.86 cells to the soluble fraction of crude bovine liver extract. SU.86.86 (2×10^5) in serum-free RPMI-1640 media were added to the upper compartment of the chamber; lower wells contained serum-free RPMI-1640 as control, and the soluble fraction of crude bovine liver extract (0.01–1,000 μg/ml). **(B)** Haptotactic response of SU.86.86 cells to the insoluble fraction of crude liver extract. The filter coated with insoluble fraction of crude liver extract (0.01–10,000 μg/ml) was placed between the upper and lower compartments of the chamber. In chemotaxis and haptotaxis assays, cells that had migrated to the lower side of the filter were counted by light microscopy under high-power fields (×200). Results were expressed as the number of migrated cells and represented as the mean ± SD of five experiments, each assayed in quadruplicate.

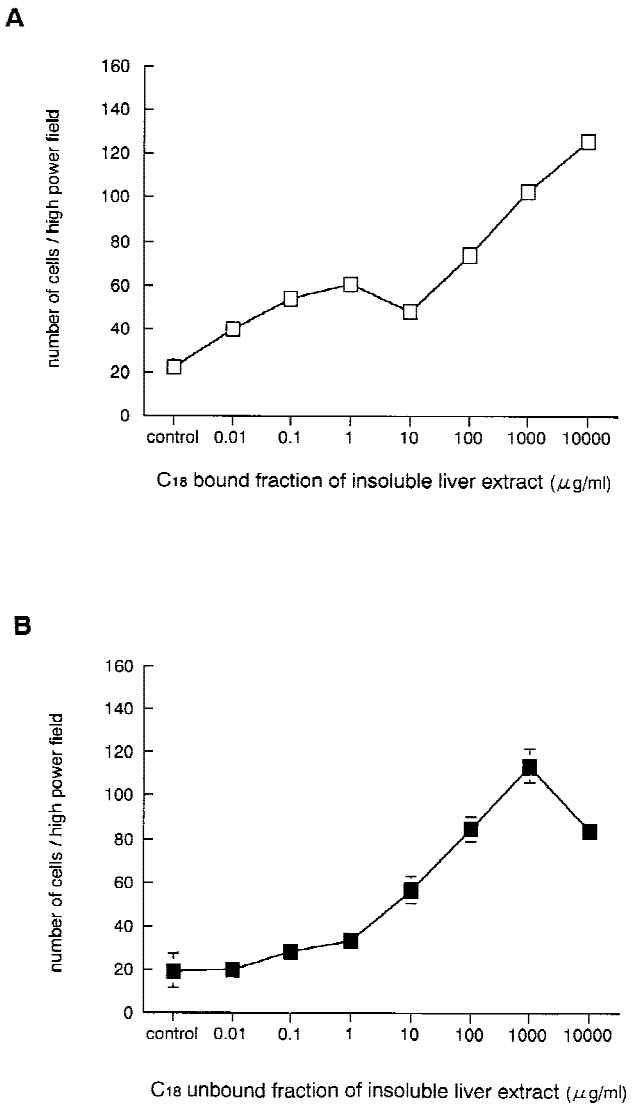

Fig. 2. **(A)** Haptotactic response of SU.86.86 cells to C_{18} bound fraction of insoluble liver extract. **(B)** Haptotactic response of SU.86.86 cells to C_{18} unbound fraction of insoluble liver extract. The filter of chemotaxis chamber was coated with the C_{18} bound (A) or unbound (B) fraction of insoluble liver extract according to the procedure described under Materials and Methods.

DISCUSSION

The process of metastasis includes multistep events involving multiple host–tumor interactions. The final step of metastasis is proliferation as a secondary colony in the target remote organ. On the other hand, in the case of organ-specific metastasis, cooperation between tumor cells factors (''seed'') and host organ factors (''soil'') is essential to achieve the metastasis [8]. A number of studies on the factors expressed by tumor cells, such as adhesion molecule, protease, growth factors, and extracellular matrix, contribute to metastasis [6,9]. For example, CD44 expression is related to tumor progression in colorectal cancer [10], cathepsin D protease expression is a prognostic factor in human breast cancer and ovarian cancer [11,12] and vascular endothelial growth factor (VEGF) facilitate angiogenesis and induce tumor progression [13,14]. However, fewer studies on the organ-derived factors (''soil'') relate to organ-specific metastasis.

Hujanen and Terranova [15] reported that extracts of soluble components from various organs induce different migration of organ-specific tumor cells. Cerra and Nathanson [16] found organ-specific chemotactic factors present in lung extracellular matrix. They also showed that chemotactic activity occurred in murine liver extracellular matrix [17]. These investigators prepared extracellular matrix from murine liver extracted with 4 M guanidine and examined the solubilized ingredients for chemotactic activity. We prepared bovine liver extract according to their procedure with some modification. In the present study, SU86.86 cells, a metastatic pancreatic cancer cell line that had been raised from liver metastasis site, failed to demonstrate any chemotactic response to the soluble fraction of liver extract (Fig. 1A). Soluble chemoattractants for metastatic cancer cells in the host organ are thought to act as chemoattractants mainly at the step when cancer cells attach to endothelium of a remote organ and migrate toward subendothelial tissue. Interaction of adhesion molecules between cancer cell and endothelium of host organ may play an important role in this step [18]. Once metastatic cancer cells are attached to the endothelium, however, interaction between cancer cells and the extracellular matrix of the host organ increases its importance for cancerous progress to a deeper site [19]. This movement of cancer cells migrating into deeper sites of host organs appears to be haptotactic, rather then chemotactic. In our study, SU.86.86 cells showed great haptotactic migration toward the insoluble fraction of liver extract (Fig. 1B). This haptotactic migratory curve indicates that the insoluble liver extract contains multiple biological ingredients and that each substance has a proper concentration range that acts as a chemoattractant for SU.86.86 cancer cells.

Purification of the insoluble fraction of liver extract is an arduous procedure because of its insolubility. We separated the liver extract with a C_{18} column according to the reverse-phase chromatography procedure. Alkyl saline C_{18} is a strong adsorbent for protein that will adsorb almost all proteins, and only small proteins or peptides can pass through the C_{18} column without being trapped. Regardless of the denatured condition, C_{18} bound and unbound fractions of insoluble liver extract induced haptotactic response in SU.86.86 cells in a concentration-dependent manner (Fig. 2A,B). The two haptotaxis curves for C_{18} bound and unbound fractions showed different figures: biphasic and parabolic, respectively. Each fraction must be composed of different biochemical ingredients. Furthermore, these results suggest

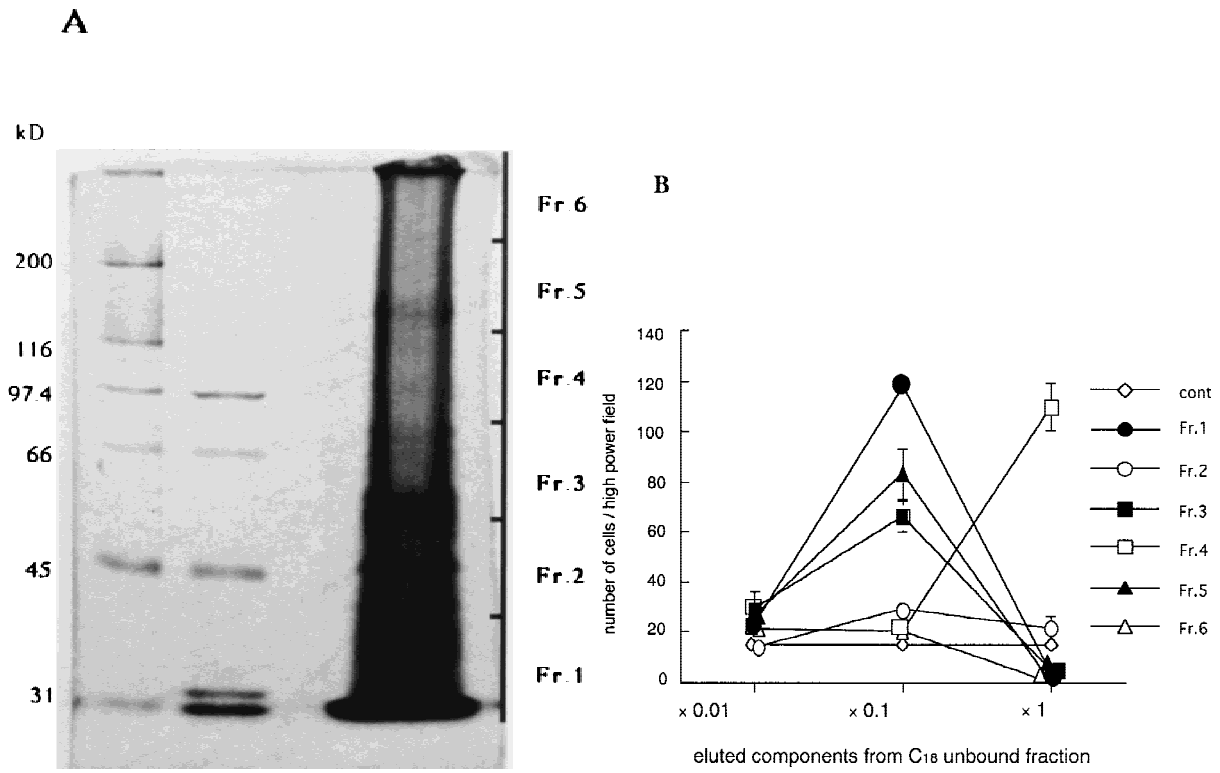

Fig. 3. **(A)** The C_{18} unbound fraction of insoluble liver extract was separated on the 8% polyacrylamide gel by electrophoresis. First and second line from the left side of gel is control marker of high molecular weight and low molecular weight, respectively. After electrophoresis, the gel was divided into six pieces according to the indicated molecular weight, and proteins were eluted from the gel portions by electroelution. The gel was stained with Coomassie blue. **(B)** Chemotactic migratory response of SU86.86 cells to six protein fractions eluted from the gel pieces. Molecular-weight range of each fraction (Fr. 1–Fr. 6) was <40 kD, 40–60 kD, 60–80 kD, 80–120 kD, 120–210 kD, and >210 kD, respectively.

that biochemical ingredients in liver extract acting as chemoattractant may not be a type of bioactive enzyme such as protease, growth factor, and cytokine, which require preservation of their native conformation, but that they may be polypeptides or proteins that contain bioactive peptide sequences, such as argenine-glycine-aspartic acid (RGD) sequence [20], in their primary structure.

The C_{18} unbound fraction of insoluble liver extract was composed of peptide and proteins of lower molecular weight, which were relatively soluble in ordinary buffer solutions. Soluble components are capable of displaying their activity as chemoattractants by chemotaxis instead of haptotaxis. The C_{18} unbound fraction was dissolved in SDS/sample buffer and separated by SDS-PAGE (Fig. 3B). Numerous dark bands were seen in areas of relatively lower molecular weight on the gel. The six elutriated protein fractions induced different chemotactic responses in SU.86.86 cells (Fig. 3B). In particular, SU.86.86 cells showed considerable migration toward the Fr. 1, which contained proteins of lower molecular weight (<40 kD), suggesting that some degraded bioactive products might exhibit chemoattractivity. Among the elutriated protein fractions, Fr. 1, Fr. 3, and Fr. 5 induced chemotactic migratory response in SU.86.86 cells at concentrations of about 2 μg/ml, and inhibit response at concentrations 10 times higher. One of the reasons for this inhibition may be that we are incapable of completely removing toxic substances such as SDS from eluted fractions by dialysis. Incidentally, partially purified liver extracts for chemotaxis assay contain relatively large amounts of chemoattractants compared with crude extracts for haptotaxis assay. Furthermore, even if chemoattractants in haptotaxis and chemotaxis are identical, receptors against chemoattractants on the cell surface might be different, inducing different migratory responses [20].

In summary, in the insoluble fraction of bovine liver extract, we found chemoattractive ingredients that induced haptotactic migration of liver-metastatic pancreatic cancer cells. These chemoattractants are conceived to be degraded products of extracellular matrix, containing bioactive peptide sequences because (1) the chemoattractants induced motility of tumor cells in their denatured condition; and (2) the chemoattractants, derived from bovine liver extract, induced motility of human cancer cells in a trans-species manner. Almost all cancer cells produce or induce protease to invade surrounding tissue, degrading extracellular matrix [19,21–23]. Their

degraded products, containing bioactive components, soluble or insoluble, may play an important role in the development of cancer metastasis [24]. Furthermore, interaction between cancer cells and degraded ingredients of the extracellular matrix in the target organ may contribute to organ specificity of cancer metastasis [25]. Precise understanding of the relationship between cancer (''seed'') and target organ (''soil'') will give us novel strategies for cancer therapy [26].

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